THE RÔLE OF THE LYMPHATIC SYSTEM IN THE PATHOGENESIS OF ANTHRAX

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WHEN animals die of anthrax there are prominent pathological changes in the lymphatic system. If the primary site of infection is the skin, the regional lymph nodes are enlarged, haemorrhagic and contain living anthrax bacilli. At the primary lesion there is oedema and cellular infiltration, as well as multiplication of bacilli (Cromartie, Bloom and Watson, 1947). If, on the other hand, anthrax is initiated by the inhalation of spores there is less involvement at the site of invasion. Changes in the lung parenchyma have been described, such as hyperaemia, oedema and cellular infiltration in animals sacrificed at various intervals between 12 and 48 hr. after exposure to a dose expected to be fatal (Young, Zelle and Lincoln, 1946), but these are certainly slight and may be absent (Ross, personal communication). Barnes (1947) has shown that bacilli do not multiply in the lung itself, but cause a massive infection of the mediastinal lymph nodes which precedes bacillaemia and death, an observation confirmed by plate counts on a suspension of the nodes. He concludes that the alveolar lining acts merely as a portal of entry of the bacilli, and that multiplication and subsequent invasion of the blood stream takes place only after infection of the lymph nodes draining the lungs.

These conclusions as to the rôle of the lymph nodes in the pathogenesis of anthrax infection are based mainly on post-mortem and histological investigations, and on counts of bacilli in the tissues at various stages of the disease. An alternative approach is to measure the output of bacilli from infected tissues into the lymph and blood streams, and this has been done in the experiments described in this paper.

METHODS

Pulmonary anthrax

Rabbits were exposed to a passing cloud of single *Bacillus anthracis* spores, using the apparatus described by Henderson (1952). The strain used was M36, which is highly virulent for rabbits. The exposure time was one minute, and the spore concentration varied from 0·9 to 1·5 $\times$ 10^6 spores/litre in different experiments. Assuming a minute volume of 400 ml., 3·6 to 6 $\times$ 10^5 spores would be inhaled; of these, 20–25 per cent would be retained in the lungs (Barnes, 1947). This is sufficient to kill 80–100 per cent of the exposed rabbits, death usually occurring 2 to 5 days after infection.

At various times after exposure, samples of right lymphatic and thoracic duct lymph were collected. The rabbits were anaesthetised with pentobarbitone sodium 32–40 mg./kg. The cervical thoracic duct was cannulated with polyethylene tubing, or with a fine metal cannula attached to such tubing; both were initially filled with sterile heparin solution, 10 mg./ml. If attempts to cannulate the duct failed, lymph was collected from the cut duct by pipette.

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No difference in results between the two methods was found. The flow from the thoracic duct was measured at hourly intervals up to 14 hr. after cannulation ; anaesthesia was maintained during this time.

The right lymphatic duct is difficult to cannulate in the rabbit, since it is a small fragile vessel buried in fat. It was therefore cut and the lymph flowing from it collected by pipette for 15 min. In view of the difficulty in obtaining right duct lymph, in some rabbits the duct was ligated. This we consider diverted the right duct lymph to the thoracic duct. (A description of the operation and the reasons for this conclusion are given elsewhere (Hughes, May and Widdicombe, 1956)). After 2–3 weeks the rabbits were exposed to anthrax and, by subsequent cannulation of the thoracic duct, lymph was collected from the drainage areas of both ducts.

Lymph was plated on tryptic meat agar after suitable serial dilutions, and the plates incubated at 37°. Lymph and blood were plated every 1–2 hr.

Anthrax infection of the fore-foot

Rabbits are so susceptible to the M36 strain of anthrax when it is injected subcutaneously that only a few spores are needed to cause death and the dose cannot be measured accurately. A less virulent strain " Vollum " was therefore chosen, of which the subcutaneous LD_{50} is about 400 spores. The choice of injection site depended on the lymphatic drainage : the middle of the fore-foot was used, between the metacarpals. This site is drained by three or four lymph vessels in the lower limb, which meet and anastomose round the brachial artery before entering the axillary lymph node : this drains into the subclavian duct which is easy to cannulate. In some rabbits, part of the lymph from the foot passes to the pectoral and scapular lymph nodes and then to the subclavian duct. These observations were made in a number of rabbits by injection of Evans Blue solution into the foot followed by post-mortem dissection.

The subclavian lymphatic duct was cannulated with fine polyethylene tubing under pentobarbitone or thiopentone sodium anaesthesia, usually the latter. In survival experiments the rabbit was prevented from tearing out the cannula by passing the tubing behind the *manubrium sterni* and beneath the skin of the left side of the neck to appear at the back of the neck. The rabbit was placed in an open-mesh metal box ($14 \times 4\frac{1}{2} \times 4\frac{1}{2}$ in.)($35{\cdot}6 \times 11{\cdot}4 \times 11{\cdot}4$ cm.) with food and water. During the night the lid of the box was down, so that the rabbit remained in the prone position and its only movements were to readjust its position and to eat and drink. During the day the lid of the box was usually removed so that the rabbit could sit up, but it was not allowed to walk about.

Injection of anthrax spores was made into the foot either immediately after or at various intervals before cannulation of the subclavian duct. The dose was usually about 800 Vollum spores in 0·2 ml., which is about an LD_{80}. At intervals of $\frac{1}{2}$ to 2 hr. the flow rate was recorded and lymph and blood plated (after dilution if necessary) ; this routine was broken at night, when the rabbits were left undisturbed with the lymph draining.

RESULTS

Pulmonary anthrax

In preliminary experiments, rabbits were exposed to a cloud of anthrax spores and at various intervals afterwards samples of lymph were collected for 15 min. from the thoracic and right lymphatic ducts. We tried to do this early in the course of bacillaemia before the terminal stages were present, but owing to the variability of the time course of anthrax in rabbits this was only successful in 12 out of 27 experiments.

With two exceptions the anthrax counts of the blood ranged from 5 to 75 organisms/ml., whereas the lymph samples usually gave counts of several thousand per ml. The abdominal lymph stream was not the source of anthrax bacilli in the cervical thoracic duct, since only in the terminal stages of anthrax did the abdominal lymph give positive cultures. We conclude from these results that, after the stage of mediastinal lymphadenitis, there is a massive exit of

bacilli from the lymph nodes into the thoracic and right lymphatic ducts, and that this occurs in the early stages of bacillaemia when the blood count is relatively low. Thoracic duct lymph contained 250–5,000 organisms/ml.: since the output of lymph is about 3 ml./hr. in the anaesthetised rabbit with pulmonary anthrax, the duct is discharging 750–15,000 organisms/hr. The output of the right lymphatic duct was more difficult to determine since the samples were small and the rates of flow known only approximately. But, assuming that the right lymphatic duct drains at least three-quarters of the lungs and mediastinal lymph nodes (Warren and Drinker, 1942), it is likely that at this stage of the disease up to 60,000 organisms per hour are entering the blood. The blood counts of 5–75 organisms/ml. correspond to a total of 600–10,000 for the whole blood volume.

If the main source of the bacillaemia is bacilli from the lymph it should be possible to prevent or delay this by preventing the infected lymph from entering the blood stream. Using the preparation in which right duct lymph was diverted to the thoracic duct, the latter was cannulated 18–30 hr. after inhalation of spores. Lymph was drained for as long as possible after cannulation (up to 14 hr.) and in this way it was hoped to compare the times of onset of lymph and blood infections. The extreme difficulty of timing the experiments was again apparent. The criterion of success was that both blood and lymph should be sterile at the start of the experiment and that one or both should become infected during its course. This occurred in 3 and partially in 1 out of 18 experiments, the results of which are shown in Table I. In experiments 1, 3 and 4 both blood and lymph were sterile at the start and both became infected during the experiment; the lymph infection preceded the blood infection by 2–4 hr. and in the last 2 experiments the lymph counts were far higher than those in the blood. In experiment 2 the bacillaemia was present initially whereas the lymph was sterile but later became infected. Of the remaining experiments, in 8 both blood and lymph were infected before cannulation and in 6 neither fluid became infected during the experiment.

TABLE I.—*Appearance of Anthrax Bacilli in Blood and Thoracic Duct Lymph after Inhalation of Spores*

Experiment.	Time after exposure (hours).	Lymph count (orgs./ml.).	Blood count (orgs./ml.).
1	26	0	0
	27	*ca.* 150	0
	31	*ca.* 175	*ca.* 100
2	30	0	40
	33	*ca.* 50	15
3	32	0	0
	33	15	0
	35	1,225	40
4	24	0	0
	26	50,000	0
	27	200,000	204

The right lymphatic ducts had been previously ligated. Samples of blood and lymph were cultured at hourly intervals. Selected results are given, corresponding to the last time when both fluids were sterile, the first appearance of bacilli in one fluid, and the presence of bacilli in both.

Anthrax infection in the fore-foot

In our first experiments we examined lymph early in the stage of bacillaemia. The subclavian lymphatic duct was first cannulated and a 15 min. sample obtained. Then, one or two lymphatics in the lower part of the limb were dissected out and cut, and a 15 min. sample of lymph collected.

Fifteen experiments were done, of which 6 showed no lymph infection; the results of the other 9 are given in Table II. In all but 2 experiments there was a greater output of bacilli in the efferent lymph than in the afferent, the difference sometimes being several thousand-fold. Afferent lymph was collected from only one or two out of several lymphatics draining the site of infection so these figures should be multiplied by 2 or 3 to apply to the entire lymph drainage of the foot. During the collection of lymph the node was not massaged (or approached in dissection); on the other hand flexion and extension of the ankle joint inevitably massaged the site of injection, and this may have liberated organisms into the afferent lymph. It is therefore probable that the anthrax counts on afferent lymph are higher than they would be in the quiescent limb.

In the first 6 experiments shown in Table II a large dose, *i.e.*, 8,500 spores, was injected; this was followed by anthrax infection at the site of injection, shown by the presence of oedema and confirmed by culturing the tissue (experiments 5 and 6). In experiments 7, 8 and 9 there was slight or no swelling of the foot and bacilli were discovered in only 2 rabbits by culturing the tissues of the foot; no anthrax bacilli were found in the afferent lymph. The positive cultures from the foot in experiment 8 could be due to the considerable bacillaemia.

TABLE II.—*Living Anthrax Bacilli in Lymph from the Fore-limb after Injection of Spores into the Foot*

Experiment.	Time from injection (hours).	Dose (spores).	Efferent lymph (orgs./hr. $\times 10^{-3}$).	Afferent lymph (orgs./hr. $\times 10^{-3}$).	Axillary lymph node.	Blood (orgs./ml.)	Injection site.
1	24	8,500	1,500	3	Confl.	35	—
2	25	8,500	5	15	+	0	—
3	28	8,500	2,700	1	Confl.	266	—
4	29	8,500	1	4	++	0	—
5	48	8,500	214	0	Confl.	35	Confl.
6	49	8,500	5	0·4	,,	1,320	,,
7	29	850	6	0	,,	0	,,
8	46	850	28,300	0	,,	Confl.	,,
9	47	850	49	0	,,	430	0

Efferent lymph was from the cannulated subclavian duct, afferent lymph from two or three lymphatics in the lower limb. Confl. = confluent growth.

In a second series of experiments lymph was collected from the subclavian duct in the *unanaesthetised* rabbit; the experiments usually continued for about 48 hr. Table III gives 4 results in which positive lymph cultures were obtained. Three of the rabbits had outputs of many millions of organisms per hour. Experiment 2 is of particular interest since over 30 million organisms were collected in the subclavian lymph during more than 7 hr. while the blood remained sterile. In experiment 4 the lymph discharged 10,000–30,000 organisms/hr. for nearly 5 hr. while the blood remained sterile. In experiments 2 and 4

the tissues of the foot were oedematous and positive cultures were obtained; this was not seen in experiments 1 and 3.

TABLE III.—*Output of Anthrax Bacilli in Subclavian Lymph in Unanaesthetised Rabbits during Infection of the Fore-foot*

Experiment.	Time from injection (hours).	Duration of samples. Hr.	Min.	Lymph anthrax (orgs./hr. $\times 10^{-6}$).	Blood anthrax (orgs./ml.).
1	24	3	20	1·1	3,000
	27	1	45	6·7	7,500
2	23	18	0	*ca.* 1·0	0
	41	7	0	4·7	0
	48	16	0	7·3	50,000
3	32	0	35	18·2	14,000
4	30	17	0	*ca.* 0·005	0
	47	1	45	0·01	0
	49	3	0	0·03	0

Abridged results from 4 experiments. Average results from several samples are given in each line, with the period over which the samples were collected and time from injection of the beginning of the period. The blood anthrax counts were done at the end of each period.

DISCUSSION

Our results show that before bacillaemia can be recognised the lymph nodes discharge bacilli into the thoracic and right lymphatic ducts; there may be an output of 60,000 organisms per hour while the blood anthrax count is still low. The change from sterile lymph to highly infected lymph is rapid: in three to four hours the counts may rise to 200,000 organisms/ml. Young *et al.* (1946) showed that when guinea-pigs were exposed to a cloud of anthrax spores (the dose rather less than LD_{50}) organisms almost immediately reached the peribronchial lymph nodes and remained there with little change in number for 48 hr. Our rabbits had a considerably higher dose (LD_{80-100}) but the results were similar. Presumably the bacilli multiply in the lymphoid tissue but remain there without appreciable liberation into the blood stream until a critical point is reached and a progressively increasing output of organisms occurs.

The experiments also show that the right lymphatic and thoracic ducts are not the only source of blood bacilli. There might be lymphatic channels opening into the blood stream other than the two main ducts, but dissections suggest that they must be very small. The view that bacilli may enter the blood stream directly from tissues has been put forward by Boquet and Saenz (1931) and by Buchner (1888), but there is no direct support for it. In perfused lungs which had been previously exposed to anthrax spores we found that the blood recirculating through the lung vessels did not become infected. It is most likely that bacilli enter the blood stream directly from the engorged lymph nodes. It is supported by the closeness in time of onset of blood and lymph infections: we may suppose that when the nodes become sufficiently engorged with organisms, bacilli escape both into the efferent lymph and into the blood sinuses.

Unlike the lungs, the tissues of the foot support the multiplication of anthrax bacilli, but the rôle of this in the production of bacillaemia is small in relation to that of the regional lymph nodes. With an injection of about LD_{80} there may be no macroscopic evidence of local infection and there may be no bacilli in a 15 min. sample of afferent lymph. At the same time many millions of bacilli may be escaping from the regional lymph nodes. When larger doses of spores are injected a local lesion is evident, but even when the oedematous foot is massaged there is a far greater number of bacilli in the efferent than in the afferent lymph. The bacilli in the efferent lymph are newly formed in the node, and not passing through from the foot, since the lymph node of the normal rabbit is a highly efficient filter of anthrax bacilli (Widdicombe, Hughes and May, 1955) and inflamed nodes are even more efficient in this respect than normal ones (Smith and Wood, 1949). Multiplication of bacilli in the foot therefore contributes little to the bacillaemia, either because germination of the spores is delayed, or because the foot supports multiplication of bacilli far less readily than the lymph nodes, or because the bacilli are fixed to the tissues and do not appear in the lymph.

In two experiments of this series highly infected subclavian lymph was collected by drainage for several hours while the blood samples remained sterile so that there was no appreciable entry of bacilli into the blood by routes other than the lymph. With pulmonary anthrax, lymph infection preceded bacillaemia by a few hours in three out of four experiments, and cannulation of the appropriate lymph channels only delayed bacillaemia for a short time. Different strains of *B. anthracis* were used in the two types of experiment. With strain M36 in the pulmonary series it is possible that bacilli passed directly into the blood stream from the lymph nodes at about the same time as they entered the efferent lymphatics. With the "Vollum" strain (foot series) the less virulent organisms might have produced a different lymph node response and have entered the lymph stream in preference to the blood sinuses. In addition, the fact that the anthrax counts in lymph draining the mediastinal nodes were considerably smaller than those from subclavian lymph may be due to the different strains of bacilli. Since in both areas involvement of the lymph nodes is an invariable preliminary to bacillaemia and death, it seems highly probable that for reasons unknown the lymphatic system supports the growth of anthrax bacilli more readily than other tissues. Any defensive rôle of the nodes is rapidly eclipsed by the fact that they provide a good environment for multiplication of anthrax bacilli.

SUMMARY

The output of anthrax bacilli from the lymph nodes of infected rabbits has been measured. The animals were infected either by inhalation of anthrax spores or by an injection of spores into the fore-foot. The lymphatics draining the regional lymph nodes were cannulated and the lymph cultured. Several thousand to many million organisms per hour were discharged into the efferent lymph stream in the early stages of bacillaemia.

Cannulation of the efferent lymphatics draining the infected regional lymph nodes sometimes delayed for a few hours the onset of bacillaemia, but did not prevent it.

With anthrax infection of the foot a massive discharge of bacilli from the lymph nodes can occur while few or no organisms are appearing in the lymph draining the primary site of infection; macroscopic evidence of infection in the foot may be absent.

It is probable that anthrax bacilli multiply more readily in the lymph nodes than in other tissues, and that the nodes act as centres for the proliferation and dissemination of anthrax bacilli leading to bacillaemia and death.

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